Hyperlipidemia and Atherosclerotic Lesion Development in LDL Receptor–Deficient Mice Fed Defined Semipurified Diets With and Without Cholate

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Abstract—Past studies of atherosclerosis in mice have used chow-based diets supplemented with cholesterol, lipid, and sodium cholate to overcome species resistance to lesion formation. Similar diets have been routinely used in studies with LDL receptor–deficient ($LDLR^{-/-}$) mice. The nonphysiological nature and potential toxicity of cholate-containing diets have led to speculation that atherogenesis in these mice may not accurately reflect the human disease process. We have designed a semipurified AIN-76A–based diet that can be fed in powdered, pelleted, or liquid form and manipulated for the precise evaluation of diet–genetic interactions in murine atherosclerosis. $LDLR^{-/-}$ mice were randomly assigned among 4 diets (n=6/diet) as follows: 1, control, 10% kcal lipid; 2, high fat (40% kcal), moderate cholesterol (0.5% by weight); 3, high fat, high cholesterol (1.25% by weight); and 4, high fat, high cholesterol, and 0.5% (wt/wt) sodium cholate. Fasting serum cholesterol was increased in all cholesterol-supplemented mice compared with controls after 6 or 12 weeks of feeding ($P<0.01$). The total area of oil red O–stained atherosclerotic lesions was determined from digitally scanned photographs. In contrast to the control group, all mice in cholesterol-supplemented dietary groups 2 to 4 had lesions involving 7.01% to 12.79% area of the thoracic and abdominal aorta at 12 weeks ($P<0.002$, for each group versus control). The distribution pattern of atherosclerotic lesions was highly reproducible and comparable. The histological features of lesions in mice fed cholate-free or cholate-containing diets were similar. This study shows that sodium cholate is not necessary for the formation of atherosclerosis in $LDLR^{-/-}$ mice and that precisely defined semipurified diets are a valuable tool for the examination of diet–gene interactions. **(*Arterioscler Thromb Vasc Biol.* 1999;19:1938-1944.)**

Key Words: atherosclerosis ■ LDL receptor ■ dietary lipids ■ cholesterol ■ mice

The development of murine models defective in genes controlling lipid metabolism and lipoprotein expression provides an opportunity to understand better the complex interactions between diet and genetics in atherosclerosis. In the last several years, embryonic stem cell and transgenic technologies have been used to alter the expression levels of various genes affecting lipoprotein metabolism and have led to the development of murine knockout and transgenic models of atherogenesis. The ApoE knockout ($ApoE^{-/-}$),[1,2] LDL receptor knockout ($LDLR^{-/-}$),[3] and human ApoB transgenic mice[4,5] develop lesions throughout the arterial tree. Their distribution pattern and morphological features share many similarities with human atherosclerosis, suggesting that similar pathogenic mechanisms may be involved.[6,7] $ApoE^{-/-}$ mice develop hypercholesterolemia and atherosclerotic lesions spontaneously, and this can be accelerated by feeding a Western-type diet.[1,6] In contrast, $LDLR^{-/-}$ mice fed a chow diet have only a 2-fold elevation in plasma cholesterol compared with control mice and do not develop significant lesions in the first 6 months of life.[3] When fed a diet consisting of 1.25% cholesterol, 7.5% cocoa butter, 7.5% casein, and 0.5% cholic acid, these mice develop marked hypercholesterolemia and lesions throughout the aorta within 3 to 4 months.[8] Because hypercholesterolemia and lesion formation in $LDLR^{-/-}$ mice are readily enhanced by a diet supplemented with fat, cholesterol, and cholate, these mice provide a unique opportunity for evaluation of early events in atherogenesis.

Before the development of atherosclerosis-prone gene-targeted mutant mice, many studies were performed with normal mice fed chow-based diets supplemented with varying amounts of saturated fats, cholesterol, and cholate to

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induce atheromatous lesions. In particular, C57BL/6 mice are susceptible to dietary intervention and develop foam cell–rich lesions in the aortic root, but not advanced atheromas.[9–13] Dietary cholate was required to achieve significant hypercholesterolemia, presumably by interfering with hepatobiliary excretion of cholesterol. Most published studies of atherosclerosis in the LDLR$^{-/-}$ mice have relied on similar diets supplemented with cholate, cholesterol, and lipid that were used in the earlier C57BL/6 mouse studies. This has led to criticisms of the LDLR$^{-/-}$ mouse model based on the speculation that toxic metabolic effects of cholate may modify the pathogenesis of vascular disease in ways not relevant to human atherosclerosis. For example, cholate may cause hepatic steatosis that can progress to cirrhosis accompanied by several host metabolic, physiological, and hormonal changes that can potentially interfere with the interpretation of studies focusing on the histopathological and molecular events during atherogenesis. Recent data from our group and others indicate that cholate is not necessary and that a diet supplemented with cholesterol and saturated fat is sufficient for aortic lesion development in LDLR$^{-/-}$ mice.[14,15]

From a nutritional perspective, the dilution of a chow diet with purified lipids, such as hydrogenated coconut oil, increases the caloric density of the diet and reduces the ratio of essential nutrients to dietary energy, thereby potentially contributing to marginal nutrient intake in mice consuming the atherogenic diet. Chow diets do not take advantage of the accumulated knowledge concerning nutritional requirements of mice and the experience of many investigators using precisely controlled semipurified or purified diets for studies of chronic disease processes in rodents.[16–19] Chow diets are formulated from natural ingredients to satisfy the minimal nutrient requirements for growth and reproduction but they differ individual nutrients over time, seasonally, in different geographic locations and between companies in the sources of ingredients included in the final product.[20] Furthermore, many man-made and natural toxins are detected in chow diets, such as aflatoxins, nitrosamines, pesticides, herbicides, and heavy metals.[20–22] Chow diets contain a variety of natural substances from grains, fruits, and vegetables that may modify lipid metabolism and atherogenesis, including a diverse array of soluble and insoluble fiber sources and a multitude of biologically active phytochemicals such as carotenoids and flavonoids. For example, the latter constituents may exert antioxidant actions that could influence atherogenesis and confound experiments.

We propose that investigators of atherogenesis using the many new transgenic and gene knockout models should consider using precisely defined semipurified diets in their studies. This approach adds very little to the overall costs of in vivo investigations and can help improve the quality of data obtained and the comparison of results among laboratories over time. Furthermore, the use of semipurified diets in murine studies provides a method for precise control of dietary and nutritional factors, allowing for a meaningful evaluation of specific nutritional interventions that may be relevant to human disease processes. We therefore designed and tested several semipurified diet formulations in a study of atherogenesis in LDLR$^{-/-}$ mice.

Methods

Mice

Male LDLR$^{-/-}$ mice (homozygous) from a mixed C57BL/6J×129Sv background (50% C57BL/6J-50% 129Sv) were purchased from Jackson Laboratories and maintained in the Longwood Medical Research Center facility in accordance with guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (DHEW publication No. (NIH) FS-23]. At 8 to 12 weeks of age, mice that reached a weight of 21 to 22 g were randomly assigned to 1 of 4 diets (see below) fed ad libitum for 12 weeks. For experiments that included analyses of body weight, total plasma cholesterol and triglycerides, and atherosclerotic lesion formation in the aorta, groups consisted of 6 mice. Additional male LDLR$^{-/-}$ mice were fed identical diets and killed to obtain plasma for lipoprotein analysis, liver function tests, and tissues for histology.

Diets

Four diets were used in this study. Each diet was a modification of the AIN-76A semipurified diet for mice and rats[18,19] and prepared by Dr Edward A. Ulman at Research Diets, Inc, according to our formulations (Table 1). The diets provide adequate concentrations of all known essential nutrients for the mouse. The carbohydrate component was altered from the original AIN-76A formulation by including expanded maltose dextrin, which allows the lipid concentration to vary from the range of 10% to 40% of total energy (≈5% to 20% by weight) without a problem of "settling out." Furthermore, the carbohydrate modifications allow a diet to be fed as a powder, a liquid formulation, or processed into pellets (used in this study). The 4 experimental diet groups include diet 1 group (Research Diet D12102), control (10% kcal lipid); diet 2 group (Research Diet D12107), high fat (40% kcal lipid), moderate cholesterol (0.5% by weight); diet 3 group (Research Diet D12108), high fat, high cholesterol (1.25% by weight); and diet 4 group (Research Diet D12109), high fat, high cholesterol, and sodium cholate (0.5% by weight). The addition of lipid to the baseline diet formulation is achieved by substituting fat (9 kcal/g of metabolizable energy) for carbohydrate (4 kcal/g of metabolizable energy) based on an equal amount of energy (kcal) rather than an equal weight (g). This approach is necessary to maintain a constant ratio of all other nutrients in the diet to energy. This technique of diet formulation avoids the problem of reduced nutrient content of the high-fat diets prepared by the dilution technique (ie, chow diluted with fat) or when fat is substituted for carbohydrate on the basis of weight.

Cholesterol Measurements and Liver Function Tests

Serum samples were collected for lipid analysis after overnight fasting. At 0 (initiation of the study), 6 and 12 weeks, blood was obtained from individual mice by tail-vein nicking and total serum cholesterol and triglyceride levels were determined by colorimetric assays (Sigma Chemical Co). Blood was obtained from the retroorbital plexus for analysis of plasma lipoproteins by fast protein liquid chromatography gel-filtration chromatography after 12 weeks of diet. Samples were anticoagulated with EDTA (3 mmol/L or 0.1% final) and sodium azide 0.02% was added as a preservative. To obtain a plasma volume of at least 250 μL, plasma was pooled from several mice within each group. Erythrocytes and leukocytes were removed by low-speed (400*g*, 10 minutes, 4°C) and platelets by high-speed (3000*g*, 5 minutes, 4°C) centrifugations. Plasma was stored at 4°C for <2 days. Plasma was subjected to fast protein liquid chromatography gel-filtration chromatography by using a Superose 6HR 10/30 column (Pharmacia Biotech) as was previously described.[23] Filtered plasma (200 μL) was loaded on the column and was eluted with 2 mmol/L sodium phosphate, 0.14 mol/L NaCl, 5 mmol/L Na_2EDTA, 0.02% NaN_3, pH 7.4, at a constant flow rate of 0.5 mL/min. Fractions (0.5 mL) were collected and total cholesterol, triglycerides, free cholesterol, and choline-containing phospholipids were measured on a Technicon RA1000 (Bayer Corp). Triglycerides were corrected for free glycerol by using a triglyceride blank reagent (Bayer Corp). The cholesterol and triglyceride assays were standard-

TABLE 1. Formulation for the Diets Used in This Study and Their Macronutrient Contents as Percentages of Total Energy

	Diet 1 (10% kcal fat, 0% cholesterol§§, 0% cholate§§)		Diet 2 (40% kcal fat, 0.5% cholesterol, 0% cholate)		Diet 3 (40% kcal fat, 1.25% cholesterol, 0% cholate)		Diet 4 (40% kcal fat, 1.25% cholesterol, 0.5% cholate)	
Ingredient	Grams	kcal	Grams	kcal	Grams	kcal	Grams	kcal
Formulation								
Casein†	200.0	800	200.0	800	200.0	800	200.0	800
Cystine	3.0	12	3.0	12	3.0	12	3.0	12
Soy oil‡	25.0	225	25.0	225	25.0	225	25.0	225
Cocoa butter§	20.0	180	155.0	1395	155.0	1395	155.0	1395
Corn Starch	375.0	1500	212.0	848	212.0	848	212.0	848
Malto-dextrin‖	125.0	500	71.0	284	71.0	284	71.0	284
Sucrose	200.0	800	113.0	452	113.0	452	113.0	452
Cellulose¶	50.0	0	50.0	0	50.0	0	50.0	0
Mineral Mix#	10.0	0	10.0	0	10.0	0	10.0	0
Dicalcium phosphate#	13.0	0	13.0	0	13.0	0	13.0	0
Calcium carbonate#	5.5	0	5.5	0	5.5	0	5.5	0
Potassium citrate, monohydrate#	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix**	10.0	40	10.0	40	10.0	40	10.0	40
Choline††	2.0	0	2.0	0	2.0	0	2.0	0
Cholesterol	0	0	4.5	0	11.25	0	11.25	0
Cholate	0	0	0	0	0	0	4.5	0
Total grams or kcal*	1055.0	4057	890.6	4056	897.35	4056	901.85	4056
	% kcal		% kcal		% kcal		% kcal	
Macronutrient content								
Protein	20		20		20		20	
Carbohydrate	70		40		40		40	
Lipid	10		40		40		40	
kcal/g in diet*	3.8		4.5		4.5		4.5	

*Calculations based on estimated metabolizable energy of 4 kcal/g (16.7 kJ/g) of protein and carbohydrate and 9 kcal/g (37.7 kJ/g) of lipid. The concentrations of minerals, vitamins, and fiber were adjusted to maintain a constant ratio to energy.

†Alcohol extracted casein, 99% protein.

‡Soy oil provides a minimal supply of essential fatty acids.

§We have selected cocoa butter for this study, because it is a saturated fat but has no cholesterol.

‖Malto-dextrin 10 is a component of the carbohydrate fraction that assists in maintaining the lipid fraction equally dispersed throughout the diet during shipping, storage, and feeding.

¶BW200 cellulose.

#AIN-76A mineral mixtures with the calcium and phosphate removed. Dicalcium phosphate, calcium carbonate, and potassium citrate, monohydrate are replaced, to increase phosphate and potassium relative to the original formulation.

**AIN-76A vitamin mixture.

††Choline provided as choline bitartrate.

§§Cholesterol and cholate (which do not contribute to total energy) are expressed as percent w/w.

ized with the National Heart Lung and Blood Institutes–Center for Disease Control Lipid Standardization program. Reagents for free cholesterol and choline-containing phospholipid measurements were purchased from Boehringer Mannheim (Germany) and external standards were not available for these assays.

Liver function tests were performed on serum samples by the Tufts Veterinary Diagnostic Laboratory, using an automated analyzer. These tests included serum lactate dehydrogenase (LDH), serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvate transaminase (SGPT), and serum bilirubin.

Tissue Sampling and Analyses

The surface area of aorta occupied by atherosclerotic lesions was quantified by en face oil red O staining, using an approach modified from Palinski et al.[24] Mice were killed, after 12 weeks of diet, by ether inhalation. A catheter was inserted into the left ventricle and the arterial tree was perfused with PBS (25 mL), then 10% buffered formaldehyde (40 mL, pH 7.4) at a pressure of 100 mm Hg. The entire aorta attached to the heart was dissected and placed overnight in formaldehyde. Using a stereomicroscope, the adventitial fat was dissected and the aorta was stained with oil red O as described by Nunnari et al.[25] After staining, the remaining adventitial fat was easily detected and was removed. The aorta was opened longitudinally, pinned en face on a black silicone–covered dish, and photographed while immersed in PBS. Slides were scanned into a Macintosh computer and the percent surface area occupied by oil red O–stained lesions was determined by using image analysis software (NIH Image). The aortic arch (1 mm above the aortic valve cusps to 2 mm below the ostium of the right subclavian artery), the descending thoracic aorta (extending to 1 mm above the ostium of the celiac artery), the abdominal aorta (including the bifurcation and 0.5 mm of the iliac arteries), and the total aorta were evaluated. After photography, portions of aorta that contained lesions were cross-sectioned

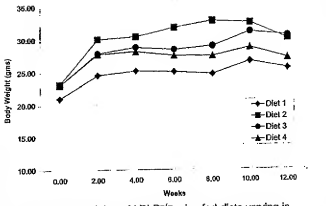

Figure 1. Body weights of LDLR$^{-/-}$ mice fed diets varying in fat, cholesterol, and cholate content. Mice were fed the diets described in Table 1 and were weighed every 2 weeks for 12 weeks. The data represent the mean weights for the 6 mice in each group at each time point.

and embedded in paraffin. Histological sections were prepared and stained with hematoxylin and eosin.

Liver slices, obtained from each animal at the time it was killed, were fixed in formalin, paraffin-embedded, and histological sections were stained with hematoxylin and eosin.

Statistical Analysis

Food intake, body weight, and serum lipids were initially analyzed by ANOVA[26] followed by Fisher's PLSD[26] to calculate pairwise comparisons among treatment groups by using Statview 4.5 (Abacus Concepts, Inc).

Results

Body Weight

The mean body weight of mice fed each of the 4 diets for 12 weeks is shown in Figure 1. Mice fed the high-fat+0.5% cholesterol diet (diet 2) showed increased body weight ($P<0.02$) compared with controls (diet 1) during weeks 2 through 10. This a common observation in studies where rodents are provided a high-fat diet, which is more palatable, resulting in a slightly greater intake of diet (kcal). However, we did not attempt to measure food intake in this study, because mice were not individually housed and they typically waste significant amounts of food when provided ad libitum. Additional effort is necessary to accurately quantitate the amount of food consumed in murine studies. Those fed a high-fat diet with higher concentrations of cholesterol or supplemented with cholate did not exhibit a weight gain that was significantly different from controls.

Lipid Analyses

The analyses of total serum cholesterol and triglyceride levels at 0, 6, and 12 weeks are shown in Table 2. A significant effect of diet on serum cholesterol was observed at 6 weeks ($P<0.0009$, ANOVA). Pairwise comparisons show that mice fed diet 1 (control diet) have significantly lower serum cholesterol than those fed the high-fat diets supplemented with 0.5% cholesterol (diet 2; $P<0.02$, PLSD), 1.25% cholesterol (diet 3; $P<0.02$, PLSD), or 1.25% cholesterol and cholate (diet 4; $P<0.0001$, PLSD). A statistically significant difference was not found between diet groups 2 and 3. However, the addition of cholate (diet 4) increased serum cholesterol compared with diets 1, 2, and 3 ($P<0.009$, for all comparisons; PLSD).

TABLE 2. Plasma Cholesterol and Triglycerides at Different Time Points

Diet	Cholesterol (mg/dL) Week 0 (Baseline)	Week 6	Week 12
1 (10% fat)	104±18	97±21	124±49
2 (40% fat; 0.5% cholesterol)	133±51	327±56*	328±111
3 (40% fat; 1.25% cholesterol)	130±37	331±46*	597±131*
4 (40% fat; 1.25% cholesterol; 0.5% cholate)	129±59	598±71*†‡	761±208*†
	Triglycerides (mg/dL)		
1 (10% fat)	52±7	50±6	63±19
2 (40% fat; 0.5% cholesterol)	40±10	85±14	110±37
3 (40% fat; 1.25% cholesterol)	50±11	58±8	141±34
4 (40% fat; 1.25% cholesterol; 0.5% cholate)	41±10	74±10	80±37

Data represent mean±SEM values for nonfasting plasma cholesterol and triglycerides.
*$P<0.02$ compared with diet group 1.
†$P<0.02$ compared with diet group 2.
‡$P<0.02$ compared with diet group 3.

Similar results were observed at 12 weeks, although variation in serum cholesterol was greater ($P<0.005$, ANOVA). Pairwise comparisons at 12 weeks show that mice fed diet 1 (control diet) have lower serum cholesterol than those fed the high-fat diets supplemented with 0.5% cholesterol (diet 2; $P<0.15$, PLSD), 1.25% cholesterol (diet 3; $P<0.007$, PLSD), or 1.25% cholesterol and cholate (diet 4; $P<0.001$, PLSD). The addition of cholate (diet 4) increased serum cholesterol compared with those fed supplemental cholesterol without cholate ($P<0.007$ versus diet 2 and $P=0.31$ versus diet 3, both PLSD). Diets did not have any significant effect on serum triglyceride levels at 6 or 12 weeks.

The analysis of plasma lipoproteins by fast protein liquid chromatography gel-filtration chromatography after 12 weeks of diet is summarized in Figure 2. The extent of lipids recovered in Superose fractions was relatively uniform and comparable in all dietary groups. Percent recovery ranged from 83% to 87% for total cholesterol, 90% to 94% for choline-containing phospholipids, and 68% to 120% for triglycerides. The data revealed that elevated total cholesterol in dietary groups 2 through 4 was the result of increased VLDL and IDL/LDL lipoproteins (Figure 2). Levels of HDL lipoproteins varied inversely with VLDL and IDL/LDL. For each lipoprotein class, levels of free cholesterol and choline-containing phospholipids were as expected, and in different dietary groups their ratios were comparable. These ratios typically were between 1 and 2 for VLDL and IDL/LDL and <0.6 for HDL (data not shown). There was no evidence for significant levels of lipoprotein X and HDL-E particles.

Development of Atherosclerotic Lesions in the Aorta

En face oil red O staining revealed minimal atherosclerotic lesion formation in mice fed diet 1 (control diet) for 12 weeks. In contrast, lesions were readily detected in each of the groups fed cholesterol-containing diets (Figure 3 and

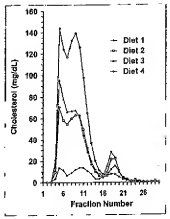

Figure 2. Plasma cholesterol profiles of LDLR$^{-/-}$ mice fed diets varying in fat, cholesterol, and cholate content. Mice were fed the diets described in Table 1 for 12 weeks, when blood samples were obtained from each dietary group and plasma was pooled. Plasma was subjected to fast protein liquid chromatography gel-filtration chromatography as described in Methods. Lipoproteins were measured in each fraction and the total cholesterol levels are plotted.

Table 3). The percent surface area of the entire aorta involved by lesions was significantly greater in mice fed diets 2, 3, and 4, compared with controls (diet 1), as well as in mice fed diet 4 compared with group 2. The interpretation was similar when the arch, thoracic, and abdominal regions were evaluated individually (Table 3). The anatomic distribution of atherosclerotic lesions was identical in dietary groups 2, 3, and 4 (Figure 3). Lesion-predisposed sites included the aortic root, the lesser curvature of the arch, and near the orifice of the brachiocephalic, intercostal, celiac, superior mesenteric, and renal arteries.

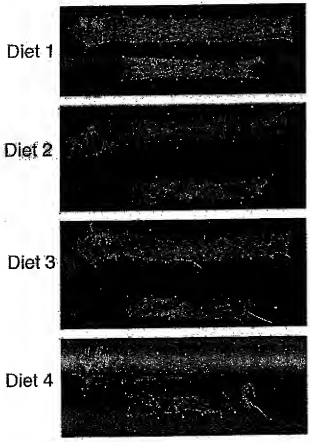

Figure 3. Oil red O–stained atherosclerotic lesions in aortas of LDLR$^{-/-}$ mice fed diets varying in fat, cholesterol, and cholate content. Mice were killed after being fed defined diets described in Table 1 for 12 weeks. Aortas were prepared and stained with oil red O as described in Methods. One representative aorta from a total of 6 in each of the 4 dietary groups is shown.

Histological examination revealed a similar morphology and cellularity in atheromas from each of the groups fed cholesterol-containing diets (Figure 4). The lesions had characteristic intimal thickening with foam cells, and apparent smooth muscle cell infiltration.

Liver Function Tests and Histology

To determine if consumption of a cholate-containing diet for 12 weeks led to liver damage, serum liver enzyme levels and liver-derived products were measured and histological sections of liver were evaluated. The liver function test results were comparable between all dietary groups, suggesting that the liver parenchyma and biliary system were not seriously damaged after 12 weeks of feeding. Of particular interest, mice in group 4 (fed 1.25% cholesterol with cholate) did not have a significant elevation in serum bilirubin, alkaline phosphatase, γ-glutamyltransferase (GGT), alanine aminotransferase (ALT), or aspartate aminotransferase (AST), or decrease in albumin when compared with group 3 (also fed 1.25% cholesterol, but without cholate) (data not shown). Hematoxylin and eosin sections of liver revealed substantial steatosis in dietary groups 3 and 4, with greater fatty changes observed in the cholate-supplemented group. There was no histological evidence of hepatocyte necrosis, apoptosis, inflammation, fibrosis, or cirrhosis at the time point examined. However, all cholate-fed mice had stones in the gallbladder, whereas none were observed in mice fed cholate-free diets.

Discussion

This study demonstrates that nutritionally defined semipurified diets are appropriate for the study of diet–genetic interactions in murine atherosclerosis. They offer several advantages compared with the commonly used chow-based diets, including reproducibility and uniformity of content, and the ability to precisely alter composition. Dietary lipid saturation and concentration are frequently the focus of hypotheses in experimental atherogenesis as a consequence of the enormous body of clinical and epidemiological data suggesting their importance in vascular disease. A semipurified diet allows the investigator to alter lipid concentration by substitution for an equivalent amount of energy from carbohydrate, to maintain a constant ratio of all other nutrients to energy in the control and high-fat diets. This is impossible to achieve when adding fat by dilution to a chow diet. The dilution technique confuses the interpretation of results. Indeed many investigators using diets prepared by dilution of chow with fat are seemingly unaware of the fact that mice consuming an identical amount of energy from the high-fat diet are also exposed to a significantly lower amount of all components of the chow, such as protein, all vitamins and minerals, and biologically active but nonnutrient factors such as fiber and phytochemicals, including those with antioxidant properties. The role of specific vitamin and mineral deficiencies or excess can be precisely examined by using semipurified diets

TABLE 3. Atherosclerotic Lesion Formation in Mouse Aortas After 12 Weeks of Diet

Dietary Group (n=6)	Percentages of Aortic Surface Area Involved by Oil Red O–Stained Lesions			
	Total	Arch	Thoracic	Abdominal
1 (control)	0.16±0.32	0.10±0.19	0.08±0.17	0.67±0.94
2 (0.5% cholesterol)	7.02±3.97‡	24.96±12.09§	2.80±2.35*	5.45±4.17*
3 (1.25% cholesterol)	8.27±3.59§	31.66±11.93§	3.57±3.50*	4.10±2.90*
4 (1.25% cholesterol+cholate)	12.79±4.80§‖	34.62±15.24§	10.30±3.63§‖	5.69±5.15*

*$P<0.05$; ‡$P<0.003$; §$P<0.001$, compared with group 1.
‖$P<0.05$, compared with group 2.

because their contents can be individually manipulated in the AIN vitamin and mineral formulations.[18,19] The use of standardized formulations will allow investigators to compare data derived from different laboratories without the concern that unquantifiable differences in the chow diets used contributed to the reported results.

The semipurified formulation can be provided as a liquid or in powdered form. The liquid diet allows the investigator to obtain more precise estimates of intake because mice typically disperse much of a solid diet in a cage. Liquid diets also facilitate studies of the effects of alcohol intake and are ideal for macrophage colony-stimulating factor–deficient mice, which exhibit osteopetrosis and have no teeth, making it impossible to consume a pelleted diet.[27–29]

The effects of dietary cholate on atherosclerosis susceptibility in genetically engineered mice should be reevaluated based on our results. Mice are very resistant to the development of atheromatous lesions in the arterial tree. Historically, investigators interested in genetic differences between murine strains in susceptibility to fatty streak formation devised diets composed of chow diluted with saturated fat and supplemented with cholesterol and cholate.[10] This diet led to the discovery that the C57BL/6 strain was more susceptible to the formation of fatty streaks in the aortic root.[9] Although this dietary approach lacks many characteristics desired by experimental nutritionists, many investigators have subsequently used it in newer models of atherosclerosis developed with transgenic and gene-deletion technology. However, the potential hepatotoxic effects of cholate[11,30,31] have raised concerns that LDLR$^{-/-}$ mice fed such diets are not useful for modeling human disease.[32] Our study clearly shows that cholate is not required for the development of atherosclerotic lesions throughout the aorta in the LDLR$^{-/-}$ strain, and therefore cholate is unnecessary as a dietary additive in studies of atherogenesis in these mice. Subsequent experiments demonstrated a rapid onset of lesion formation, in that most mice fed diet 3 for 4 weeks had early lesions in the lesser curvature of the aortic arch (data not shown). Compared with mice fed diet 3 (high fat, 1.25% cholesterol), the inclusion of cholate (0.5%, wt/wt) in diet 4 caused a further increase in plasma lipids and a trend toward a greater area of the aortic surface involved by atheromatous lesions. This trend was not statistically significant because of high inter-animal variability. Cholate-fed mice also developed gallstones over the 12 weeks of investigation. It is our opinion that dietary cholate is unnecessary and perhaps a liability in studies of atherogenesis in the LDLR$^{-/-}$ mouse.

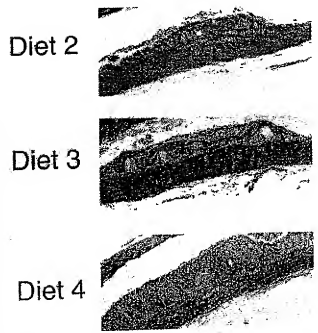

Figure 4. Histological appearance of aortic atherosclerotic lesions in LDLR$^{-/-}$ mice fed diets varying in fat, cholesterol, and cholate content. Hematoxylin and eosin–stained sections of formalin-fixed lesions from the aortas described in Figure 3 are shown.

Traditionally, cholesterol supplements of ≥1% have been used in murine and rabbit studies to enhance hyperlipidemia and the rate of lesion formation, thereby shortening the duration of studies. Diets high in cholesterol and fat may cause time- and dose-dependent hepatotoxicity, therefore lowering cholesterol concentration, may be advantageous. Our study begins to address this issue. We demonstrated that the lesion area after 12 weeks of consuming 0.5% cholesterol (diet 2) was essentially indistinguishable from mice feed cholesterol at 1.25% (diet 3). At 12 weeks of feeding, there was a trend toward higher serum cholesterol and triglycerides in diet group 3. Perhaps this would lead to accelerated lesion progression and differences in lesion area would become significant in studies of longer duration. Cholesterol levels <0.5% can induce lesions in LDLR$^{-/-}$ mice. Palinski et al[14] fed LDLR$^{-/-}$ mice for 6 months with a diet containing 21% fat and 0.15% cholesterol (without cholate) and observed extensive atherosclerotic lesion formation throughout the aorta. In evaluating aortas of retired LDLR$^{-/-}$ breeders >1 year of age, we observed lesions in the aortic arch in most (unpublished data, 1998). This indicates that LDLR$^{-/-}$

mice can develop lesions spontaneously even when fed a regular laboratory chow; however, their rate of formation is very slow, as lesions generally are not found in mice <6 months old.

The existing literature on newer models of murine atherogenesis does not allow investigators to evaluate the role of dietary lipid concentration or the source of the lipid on lesion formation. In our study, the lipid content of diet 1 (control diet) was 10% of total energy (4.3% by weight), whereas in diets 2, 3, and 4 it was 40% (20% by weight). We included soy oil at 5.5% of total energy to ensure that a supply of essential fatty acids was constant in all diets. We then manipulated cocoa butter as the variable lipid. We recommend that future investigators maintain a constant baseline supply of essential fatty acids in the diet unless they are particularly interested in this as a variable. It is possible that investigators manipulating the fat source could naively prepare or purchase a saturated fat–enriched diet deficient in essential fatty acids, which could complicate the interpretation of murine studies. Furthermore, essential fatty acid deficiency is not observed in humans except in situations of several metabolic or gastrointestinal diseases. Humans consuming diets rich in saturated fat and cholesterol easily achieve adequate intake of essential fatty acids. Therefore, murine models will more closely mimic human dietary patterns if essential fatty acid intake is adequate.

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